Genome editing of human primary keratinocytes by CRISPR/Cas9 reveals an essential role of the NLRP1 inflammasome in UVB sensing

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DOI: https://doi.org/10.1016/j.jid.2018.07.016

Accepted Manuscript

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PII: S0022-202X(18)32462-X

DOI: 10.1016/j.jid.2018.07.016

Reference: JID 1534

To appear in: *The Journal of Investigative Dermatology*

Received Date: 27 March 2018

Revised Date: 21 June 2018

Accepted Date: 6 July 2018

Please cite this article as: Fenini G, Grossi S, Contassot E, Biedermann T, Reichmann E, French LE, Beer H-D, Genome editing of human primary keratinocytes by CRISPR/Cas9 reveals an essential role of the NLRP1 inflammasome in UVB sensing, *The Journal of Investigative Dermatology* (2018), doi: 10.1016/j.jid.2018.07.016.

Genome editing of human primary keratinocytes by CRISPR/Cas9 reveals an essential role of the NLRP1 inflammasome in UVB sensing

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Keywords: keratinocytes, UVB, inflammasome, inflammation, NLRP1, CRISPR/Cas9

Short title: Ablation of *NLRP1* in human keratinocytes

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ABSTRACT

By forming a protective barrier, epidermal keratinocytes represent the first line of defense against environmental insults. UVB radiation of the sun is a major challenge for the skin and can induce inflammation, aging and eventually skin cancer. UVB induces an immune response in human keratinocytes resulting in activation and secretion of the pro-inflammatory cytokines proIL-1β and -18. This is mediated by assembly of protein complexes, termed inflammasomes. However, the mechanisms underlying sensing of UVB by keratinocytes, and particularly the types of inflammasomes required for cytokine secretion, are a matter of debate. To address these questions, we established a protocol that allows the generation of CRISPR/Cas9-targeted human primary keratinocytes. Our experiments revealed an essential role of the NLRP1 rather than the NLRP3 inflammasome in UVB sensing and subsequent IL-1β and -18 secretion by keratinocytes. Moreover, NLRP1 but not NLRP3 was required for inflammasome activation in response to nigericin, a potassium ionophore and well-established NLRP3 activator in immune cells. Since the CRISPR/Cas9-targeted cells retained their full differentiation capacity, genome editing of human primary keratinocytes might be useful for numerous research and medical applications.

INTRODUCTION

Immune cells are able to detect many different stress factors, such as pathogens or endogenous molecules released upon injury. In response to these insults, they initiate an inflammatory response, which helps to eliminate the stressors and to restore a new homeostatic state (Medzhitov, 2008). Inflammasomes represent multimeric protein complexes, which are critically involved in these processes (Strowig et al., 2012). They consist of a sensor protein, including NOD-like receptor family pyrin domain containing 1 (NLRP1), NLRP3 or absent in melanoma 2 (AIM2), the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC) and the protease caspase-1. Assembly of inflammasomes induces activation of caspase-1, which in turn cleaves and thereby activates the pro-inflammatory cytokines proIL-1β and -18 (Place and Kanneganti, 2017, Strowig et al., 2012). Secretion of these cytokines induces an inflammatory response, which is supported by a lytic type of cell death termed pyroptosis. The latter is induced upon cleavage of gasdermin D by inflammatory caspases, as the amino terminal fragment of gasdermin D forms pores in the plasma membrane upon oligomerization (Kayagaki et al., 2015, Liu et al., 2016, Shi et al., 2015). Inflammasomes are required for immunity, but play also a central role in inflammation, underlying many common (auto)inflammatory diseases (Fenini et al., 2017a, Strowig et al., 2012).

In immune cells, expression of proIL-1β, NLRP3 or AIM2 must be induced before inflammasome activation, for example by Toll-like receptor (TLR) stimulation (Latz et al., 2013). In contrast, human primary keratinocytes (hereafter HPKs) secrete IL-1β and -18 without priming upon UVB irradiation (Feldmeyer et al., 2007, Strittmatter et al., 2016b). Several stress factors, including UVB radiation (Feldmeyer et al., 2007, Hasegawa et al., 2016), cytoplasmic double-stranded DNA (Dombrowski et al., 2011), nanoparticles (Yazdi et

al., 2010), or viral infection (Reinholz et al., 2013, Strittmatter et al., 2016b), are described to induce inflammasome activation in HPKs.

Although it has been reported that HPKs do not express NLRP3 and that NLRP1 represents the important inflammasome sensor in these cells (Zhong et al., 2016), several groups suggested a role of the NLRP3 or AIM2 inflammasome in human keratinocytes (Dai et al., 2011, Dai et al., 2017, Dombrowski et al., 2011, Feldmeyer et al., 2007, Strittmatter et al., 2016b). In these studies, expression of inflammasome components was targeted by RNA interference. However, it is well known that RNA molecules can modulate the innate immune system, for example via the TLR pathway and, therefore, can possibly influence inflammasome expression and activity (Agrawal and Kandimalla, 2004, Robbins et al., 2009). Consequently, the role of specific inflammasome sensors in human primary keratinocytes should be addressed by approaches different from siRNA- or shRNA-mediated knockdown.

Interestingly, activating mutations in the gene encoding NLRP1 cause skin inflammation induced by hyperactivation of the inflammasome in keratinocytes and thereby increase the risk of developing skin cancer, demonstrating that inflammasome activation in human keratinocytes is also highly relevant *in vivo* (Zhong et al., 2016). In contrast, murine keratinocytes do neither express detectable amounts of proIL-1β protein nor assemble functional inflammasomes (Sand et al., 2018).

Isolation and propagation of keratinocytes, including epidermal stem cells, is a well-established method, which requires co-culture in the presence of mitotically-inactivated murine fibroblasts as “feeder” cells (Rheinwald and Green, 1975). Genetic manipulation of HPKs represents a useful tool for targeted gene therapy of diseases affecting keratinocytes. Recently, it has been demonstrated that epidermal stem cells from a patient suffering from junctional epidermolysis bullosa, a devastating disease caused by a defective *LAMB3* gene, can be “corrected” upon transduction with a retroviral vector encoding the wild-type protein

(Mavilio et al., 2006). Furthermore, through production of autologous epidermal sheets with the help of feeder cells, even the entire epidermis of a seven-year-old boy could be replaced by wild-type LAMB3-overexpressing keratinocytes (Hirsch et al., 2017). However, genome editing via the CRISPR/Cas9 approach would allow a more specific genetic modification of keratinocytes and, in principle, gene therapy for other genetic diseases affecting keratinocytes of the epidermis (Doudna and Charpentier, 2014, Zhang et al., 2014). In addition, direct targeting of human primary keratinocytes by CRISPR/Cas9 would represent a very useful tool for answering many scientific, medical and pharmacological questions in dermatological research.

Here, we developed and optimized a protocol for the generation of knockout HPKs with the CRISPR/Cas9 technology. Genetic modification and selection of the targeted keratinocytes was achieved by co-culture with antibiotic-resistant and proliferation-incompetent murine fibroblasts. CRISPR/Cas9-targeted keratinocytes retained the ability of differentiation and were able to form three-dimensional skin equivalents. The comparison of keratinocytes lacking either NLRP1, NLRP3 or ASC expression revealed an essential role of the NLRP1 inflammasome in UVB- and nigericin-induced IL-1β and -18 secretion.

RESULTS

Establishment of a protocol for stable genetic modification of keratinocytes

Isolation of human primary keratinocytes (HPKs) from skin biopsies (Rasmussen et al., 2013, Rheinwald and Green, 1975) or plucked hair (Aasen and Izpisua Belmonte, 2010) is well established and the culture of HPKs represents a physiologically relevant model in dermatologic research (Strittmatter et al., 2016a). Isolation and propagation of HPKs on coated or non-coated surfaces with special low-Ca^{2+} and serum-free media is simple and suppresses growth of other skin-derived cells, such as fibroblasts, Langerhans cells or melanocytes (Zare et al., 2014). However, under these culturing conditions, HPKs are prone to terminal differentiation and stop proliferation after few passages. In contrast, cultivation of HPKs in the presence of proliferation-incompetent 3T3 fibroblasts strongly increases their lifetime. In particular, the clone 3T3-J2 has been used for propagation of keratinocytes. However, these co-cultures are more elaborate than mono-cultures (Rasmussen et al., 2013, Strittmatter et al., 2016a).

Genetic manipulation of HPKs is a useful tool for dermatological research and method of choice is lentiviral transduction (Nanba et al., 2013), since transfection of plasmid DNA is highly toxic and can cause inflammasome activation (Strittmatter et al., 2016b).

We developed and optimized a protocol for the stable genetic modification of HPKs (Figure 1) based on their lentiviral transduction and co-culture with 3T3-J2 feeder cells. After separation of epidermis and dermis from a skin biopsy, keratinocytes were isolated from the epidermal layer upon enzymatic digestion with trypsin and seeded onto mitotically-inactivated (proliferation-incompetent) feeder cells in the presence of the Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor Y-27632 (day 1). Culture in the presence of this inhibitor enhances the survival and proliferation of epidermal stem cells (Nanba et al., 2013, Strudwick et al., 2015) and is therefore maintained until the first

trypsinization. After medium change (day 2), HPKs were transduced with lentiviruses at day 3 in the presence of polybrene (hexadimethrine bromide). Two days and a medium change later, the remaining feeder cells were gently removed with a diluted trypsin/EDTA solution, HPKs were detached by standard trypsinization and seeded on a new layer of puromycin-resistant and mitotically-inactivated feeder cells (day 5) in the presence of the ROCK inhibitor. The optimal density for trypsinization of HPKs was 50-60%, but not higher than 70%, as confluent cells irreversibly lose their proliferative capacity (Supplementary Figure S1). Transduction of HPKs with a lentiviral construct encoding a single guide (sg) RNA targeting the *ASC* gene and encoding Cas9 resulted in almost complete ablation of ASC expression by selecting the transduced HPKs with high concentrations of puromycin (5 µg/ml) for one week (Figure 2a and b). Prolonged selection of HPKs with puromycin rather than high concentrations for few days negatively influenced their long-term survival (results not shown).

In brief, we successfully established a protocol for the efficient stable genetic modification of HPKs. This was achieved by lentiviral transduction and selection of transduced cells with antibiotics in co-culture with antibiotics-resistant feeder cells. This protocol allows the generation of CRISPR/Cas9-mediated knockout HPKs but also of HPKs which overexpress proteins in an inducible or constitutive manner (not shown).

Characterization of knockout HPKs

Using the above-described protocol, we targeted expression of genes encoding the inflammasome proteins caspase-1 or ASC in HPKs. Western blots of the cell lysates of targeted cells revealed a dramatic reduction in caspase-1 or ASC protein expression (Figure 2c). ASC and caspase-1 expression are required for UVB-induced inflammasome activation in HPKs (Feldmeyer et al., 2007). In order to assess, whether the generated CRISPR/Cas9-

targeted HPKs are able to form a functional inflammasome, we irradiated these cells with UVB and analyzed IL-1β secretion as a readout for inflammasome activation. As expected, in ASC and caspase-1 sgRNA CRISPR/Cas9-targeted HPKs IL-1β secretion was strongly reduced compared to control cells as revealed by Western blot (Figure 2c) and ELISA (Figure 2d). This confirms earlier results obtained by siRNA-mediated knockdown experiments (Feldmeyer et al., 2007). Very importantly, control CRISPR/Cas9-targeted HPKs, which were transduced with a non-targeting sgRNA, secreted similar amounts of IL-1β as non-transduced wild-type cells. These results demonstrate that lentiviral transduction and selection of transduced HPKs by puromycin do not affect their ability to form active inflammasomes. Therefore, CRISPR/Cas9 targeting and manipulation of HPKs is a useful tool for investigating inflammasome activation in these cells.

Furthermore, we addressed the question, whether the CRISPR/Cas9-targeted HPKs retain full differentiation capacity. Ablation of ASC expression in epidermal keratinocytes of mice does not cause a spontaneous phenotype, demonstrating that it is not required for differentiation of murine keratinocytes (Drexler et al., 2012). Likewise, cultivation in the absence of EGF induced differentiation of control, caspase-1 and ASC sgRNA CRISPR/Cas9-targeted HPKs in a similar manner and, most importantly, comparable to wild-type keratinocytes (Figure 2e). Indeed, wild-type, control, caspase-1 and ASC sgRNA CRISPR/Cas9-targetd HPKs induced expression of early differentiation markers, such as keratin 1 and keratin 10, but also of filaggrin and involucrin, which are expressed in the stratum granulosum and stratum corneum, after three days in culture without EGF. When cultivated on collagen gels containing human primary fibroblasts, HPKs form a three-dimensional structure, resembling characteristic features of the epidermis *in vivo* (Pontiggia et al., 2009). In order to test, whether our protocol is compatible with stratification and three-dimensional differentiation of HPKs in skin equivalents, we seeded wild-type and ASC sgRNA CRISPR/Cas9-targeted HPKs on top of a

collagen-based dermal equivalent. After two weeks in culture, both preparations resulted in skin equivalents with a stratified epidermis-like structure (Supplementary Figure S2). These experiments demonstrate that sgRNA CRISPR/Cas9-targeted HPKs, are able to differentiate in two- and three-dimensional culture *in vitro* and, therefore, most likely, also *in vivo*.

UVB activates the NLRP1 rather than the NLRP3 inflammasome in HPKs

Recently, it has been suggested that NLRP1 is the predominant inflammasome sensor in human keratinocytes (Zhong et al., 2016). In contrast, other publications demonstrated also important roles of the NLRP3 and AIM2 inflammasomes in HPKs (Dombrowski et al., 2011, Feldmeyer et al., 2007, Hasegawa et al., 2016, Reinholz et al., 2013). An siRNA approach suggested that both NLRP1 and NLRP3 contribute to UVB-induced IL-1β secretion in HPKs (Feldmeyer et al., 2007). On the other hand, pharmacological inhibition of the NLRP3 inflammasome had no effect on IL-1β release by HPKs (Fenini et al., 2017b). To address this inconsistency, we generated NLRP1 and NLRP3 sgRNA CRISPR/Cas9-targeted HPKs, using two different sgRNAs, respectively; ASC sgRNA CRISPR/Cas9-targeted cells served as control. Gene targeting was assessed 5 days after transduction by genomic cleavage detection assays (Supplementary Figure S3). Protein downregulation was confirmed by Western blots (Figure 3a). Expression of NLRP3 can be detected only upon priming with IFNγ (Strittmatter et al., 2016b). Under this condition, NLRP3 protein expression was absent in NLRP3 sgRNA CRISPR/Cas9-targeted cells. Western blots revealed that expression of caspase-1, ASC, proIL-1β and proIL-18 was not affected by ablation of NLRP1 and NLRP3 (Figure 3a). When exposed to UVB, ASC and NLRP1 but not NLRP3 CRISPR/Cas9-targeted HPKs showed a strongly reduced inflammasome activation, even after priming with IFNγ, as reflected by the diminished IL-1β and -18 secretion (Figure 3b-d) and LDH release (Figure 3e). Nigericin is a well-established stimulus for NLRP3 inflammasome activation in immune cells (Mariathasan

et al., 2006). Interestingly, NLRP3 depletion in HPKs did not influence IL-1β release upon treatment with nigericin. In contrast, ablation of NLRP1 expression impaired IL-1β and -18 secretion in a similar manner as targeting of ASC expression (Figure 3b-d). As expected, targeting of ASC, but neither of NLRP1 nor NLRP3, impaired cytokines secretion in response to poly(dA:dT), an AIM2 inflammasome activator (Supplementary Figure S5). These experiments suggest that NLRP1 rather than NLRP3 is the key inflammasome sensor in HPKs.

DISCUSSION

By inducing inflammation, inflammasomes play a fundamental role in immunity as well as in many different common (auto)inflammatory diseases, ranging from diabetes to atherosclerosis (Larsen et al., 2007, Ridker et al., 2017, Strowig et al., 2012). Upon the detection of a broad variety of danger signals, they mount an immune response by activation of caspase-1 and in turn by release of mature IL-1β and -18. Inflammasome components are mainly expressed by immune cells upon priming, like TLR signaling, which induces expression of proIL-1β and other inflammasome components, such as NLRP3 and AIM2 (Place and Kanneganti, 2017, Strowig et al., 2012). Furthermore, IL-1β secretion can also be induced in HPKs by UVB irradiation, which does not require a specific priming signal (Faustin and Reed, 2008, Feldmeyer et al., 2007). UVB is a major threat for epidermal keratinocytes and induces inflammation, skin aging and eventually skin cancer. How HPKs sense UVB radiation is poorly understood, but studies based on si- or shRNA-mediated knockdown experiments suggested a role of both the NLRP1 and NLRP3 inflammasome (Feldmeyer et al., 2007, Hasegawa et al., 2016).

Mice are a frequently used model in biological and medical research, including skin studies. However, in contrast to HPKs, previous studies suggest that murine keratinocyte do neither express proIL-1β nor form active inflammasomes (Sand et al., 2018). In addition, caspase-1 is a regulator of UVB-induced apoptosis in HPKs but is dispensable for this process in murine keratinocytes and in the epidermis of these animals (Sollberger et al., 2015). Therefore, there are important differences concerning the role of inflammasomes in human *versus* murine keratinocytes and skin. Recently, it was reported that activating mutations in the *NLRP1* gene cause skin inflammation in humans, which is mediated by inflammasome activation in keratinocytes (Zhong et al., 2016). Since expression of NLRP3 in HPKs and in human skin could not be detected, it was concluded that NLRP1 is the most important inflammasome sensor in human keratinocytes (Zhong et al., 2016), in contrast to several other reports based

on si- and shRNA experiments (Dombrowski et al., 2011, Feldmeyer et al., 2007, Hasegawa et al., 2016, Reinholz et al., 2013, Strittmatter et al., 2016b, Watanabe et al., 2007, Yazdi et al., 2010). As it is well known that siRNA and shRNA can induce and modulate immune responses relevant for the inflammasome pathway (Agrawal and Kandimalla, 2004, Robbins et al., 2009), we targeted ASC, caspase-1, NLRP1 and NLRP3 expression by sgRNA and CRISPR/Cas9. In contrast to siRNA and shRNA approaches, genome editing of HPKs with CRISPR/Cas9 has much less severe effects on immune pathways, including inflammasomes. Most importantly, in contrast to NLRP3 sgRNA CRISPR/Cas9-targetd HPKs, those with ablated ASC or NLRP1 expression secreted drastically reduced levels of IL-1β upon UVB irradiation as well as upon treatment with nigericin compared to the corresponding control cells. This demonstrates that NLRP1 rather than NLRP3 is the main inflammasome sensor of HPKs, as also recently suggested (Fenini et al., 2017b). The importance of NLRP1 in human skin is also supported by other reports demonstrating a susceptibility to skin inflammation and autoimmunity, such as vitiligo and psoriasis, caused by variations in *NLRP1* (Ekman et al., 2014, Jin et al., 2007a, Jin et al., 2007b, Levandowski et al., 2013). It has been suggested that particularly mouse NLRP1 can directly activate caspase-1 by CARD-CARD interactions independently of ASC expression, although the presence of ASC further supports activation of the protease (Van Opdenbosch et al., 2014, Yu et al., 2018). In contrast, ASC CRISPR/Cas9-targeted HPKs did not secrete IL-1β upon UVB radiation (Figure 2c-d and Figure 3a-e), demonstrating that in HPKs ASC expression is needed for NLRP1 inflammasome activation.

Notably, we established a protocol for sgRNA CRISPR/Cas9-targeting of HPKs. HPKs represent an established model for many different research and medical applications. They are frequently used in monoculture or in conjunction with dermal fibroblasts in more sophisticated three-dimensional models, where keratinocytes form a stratified epithelium,

resembling human skin (Pontiggia et al., 2009). However, HPKs in monoculture undergo differentiation after few passages, thus limiting their applications. The use of fibroblasts as feeder cells extends the lifespan of HPKs and is the method of choice, when large amounts of HPKs are required (Rasmussen et al., 2013, Rheinwald and Green, 1975).

Recently, CRISPR/Cas9 technology was used to correct a mutation in the *COL7A1* gene causing dominant (Shinkuma et al., 2016) and recessive (Webber et al., 2016) dystrophic epidermolysis bullosa *in vitro*. Patient-derived fibroblasts or induced pluripotent stem cells (iPSCs) were targeted by CRISPR/Cas9, differentiated into keratinocytes and expanded on feeder cells.

Our approach, instead, targets directly HPKs, requires less manipulation of the cells and allows their selection. However, for the future, improvements of our protocol, particularly for gene therapeutic applications, are necessary. HPKs should be expanded after genetic modification by CRISPR/Cas9 from single cells, allowing the selection of true knockouts and even knockins and their characterization by sequencing. Lentiviral or retroviral integration can disrupt genetic information resulting in aberrant transcripts and possibly leading to cancer development (Hirsch et al., 2017, Moiani et al., 2012, Qian et al., 2017). This can be avoided using other currently available approaches, such as transfection of the recombinant Cas9 protein with the sgRNA of interest.

Here, we demonstrate successful sgRNA CRISPR/Cas9-targeting of HPKs. The modification of HPKs by CRISPR/Cas9 has the potential to result in excellent research models, for example in combination with human skin equivalents. This is strongly required due to obvious limitations of animal experimentation. In addition, several applications in human patients are conceivable, which go far beyond the treatment of epidermolysis bullosa.

MATERIALS AND METHODS

Cell culture

HEK 293T cells (ATCC CRL-3216, Manassas, VA), 3T3-J2 feeder cells (ATCC CRL-1658) and 3T3-J2 puromycin-resistant feeder cells were cultured in DMEM (Thermo Fisher Sientific, Waltham, MA) supplemented with 10% FBS (PAN-Biotech, Aidenbach, DE) and 1% antibiotic/antimycotic (A/A, Thermo Fisher Scientific). Cells were harvested with trypsin/EDTA solution (0.05%/0.02% w/v) (Thermo Fisher Scientific).

Co-cultures of HPKs and 3T3-J2 feeder cells were performed in Rheinwald and Green Medium (RGM; 3 parts DMEM, 1 part HAM's F12 Nutrient Mixture (Thermo Fisher Scientific), 10% FBS, 1% A/A, 20 µg/ml adenine (Sigma-Aldrich, St. Louis, MA), 5 µg/ml apo-transferrin (Sigma-Aldrich), 2 nM 3,3',5-triiodothyronin (Sigma-Aldrich), 200 ng/ml hydrochortisone (Sigma-Aldrich), 100 pg/ml choleratoxin (Sigma-Aldrich), 5 µg/ml insulin (Sigma-Aldrich), 10 ng/ml EGF (Sigma-Aldrich)). HPKs were trypsinized at an optimal density of 50-60%. Prior to trypsinization of HPKs, 3T3-J2 feeder cells were removed by short incubation with diluted trypsin/EDTA solution (0.005%/0.05% w/v in DPBS). HPKs were detached with trypsin/EDTA solution (0.05%/0.02% w/v) and seeded onto a new layer of mitotically-inactivated 3T3-J2 feeder cells in RGM supplemented with 10 µM ROCK1 inhibitor (Y-27632 2HCl, Selleckchem, Houston, TX). After 24 hours the medium was changed to standard RGM and HPKs were maintained in co-culture with 3T3-J2 feeder cells until experiment.

Mono-cultures of HPKs were grown in serum-free keratinocyte medium (KSFM, Thermo Fisher Scientific) supplemented with EGF and bovine pituitary extract (BPE). Cells were harvested as described above and cultured for at least 48 hours before experiment.

All cells were incubated at 37 °C in 5% CO_2 and 95% humidity.

Manipulation of cells

3T3-J2 puromycin-resistant feeder cells were generated in order to allow selection of co-cultured lentivirally-transduced HPKs. 3T3-J2 feeder cells were transduced with pLenti CMVtight Puro DEST (w768-1) (Addgene, #26430) and selected with 5 μg/ml puromycin (Sigma-Aldrich).

In order to inhibit cell proliferation, 3T3-J2 and 3T3-J2 puromycin-resistant feeder cells were treated for 2 hours at 37 °C with 10 μg/ml mitomycin C (Santa Cruz Biotechnology, Dallas, TX) diluted in growth medium. Cells were washed three times with DPBS, trypsinized as described above and plated in growth medium at a density of 50%.

Isolation of human primary keratinocytes from skin biopsies

Skin biopsies were disinfected by a short incubation with 70% ethanol and washed in DPBS. Fat was removed, and the remaining tissue cut into small pieces. The skin pieces were incubated for 2 hours in DMEM containing 1% A/A and subsequently overnight in 4 U/ml dispase II (Roche, Rotkreuz, CH) in DPBS at 4 °C. Separation of dermis and epidermis was performed the next day, and the epidermis was incubated for 20 min at 37 °C in trypsin/EDTA solution (0.25%/0.02%). A single cell suspension of keratinocytes was obtained by pipetting the epidermis up and down in DMEM containing 25% FBS and 1% A/A and passing the cell suspension through a 100 μm nylon strainer (BD, Franklin Lakes, NJ). Cells were centrifuged (170 × g, 3 min, RT), resuspended and plated onto mitotically-inactivated 3T3-J2 feeder cells (with a 5 to 1 ratio) in RGM without EGF supplemented with 10 μM ROCK1 inhibitor.

Skin biopsies were collected with informed written consent upon approval from Local Ethical Committees and were conducted according to the Declaration of Helsinki Principles.

Generation of CRISPR/Cas9-targeted human primary keratinocytes

Single guide RNA (sgRNA) were designed using the Benchling platform (https://benchling.com) and single-stranded DNA oligonucleotides (Supplementary Table S1) purchased from Microsynth (Balgach, Switzerland) were cloned into the lentiCRISPRv2 plasmid (Addgene, #52961) (Sanjana et al., 2014). Plasmids were co-transfected into HEK 293T cells with the packaging vectors psPAX2 (Addgene, #12260) and pMD2.G (Addgene, #12259) and 48 hours later lentiviruses were harvested and concentrated by centrifugation (16000 × g, 4 hrs, 4 °C). HPKs co-cultured with 3T3-J2 feeder cells were transduced 2 days after isolation. Concentrated viruses were dissolved in RGM containing 10 μM ROCK1 inhibitor and 2.5 μg/ml polybrene (hexadimethrine bromide) (Sigma). Medium was changed 24 hours after transduction to RGM containing 10 μM ROCK1 inhibitor. HPKs were splitted 4-5 days after isolation and seeded onto a fresh layer of mitotically-inactivated 3T3-J2 puromycin-resistant feeder cells in RGM containing 10 μM Y-27632. After 24 hours medium was changed to RGM containing 5 μg/ml puromycin (Sigma). Selection was performed for 48 hours. CRISPR/Cas9 targeting efficiency was assessed at the genomic level by processing the DNA with the Genomic Cleavege Detektion Kit (Thermo Fisher Sientific) or at the protein level by Western blot.

Inflammasome activation in human primary keratinocytes

For experiments, trypsinized HPKs were resuspended in KSFM supplemented with EGF and BPE and containing 10 μM Y-27632. After 24 hours medium was changed to KSFM (supplemented with EGF and BPE) and cells were grown until 70% density.

For priming, HPKs were exposed overnight to 20 ng/ml human interferon gamma (IFNγ) (Peprotech, Rocky Hill, NJ).

Prior to inflammasome activation, medium was exchanged by fresh KSFM (supplemented with EGF and BPE). HPKs were either left untreated, irradiated with 86.4 mJ/cm^2 UVB (UV802L, Waldmann, Villingen-Schwenninge, DE), stimulated with 5 μM nigericin (Selleckchem) or transfected with 2 μg/ml poly(dA:dT) (InvivoGen, San Diego, CA).

In vitro differentiation of human primary keratinocytes

HPKs were grown in KSFM (supplemented with EGF and BPE) and at 100% confluency, medium was replaced by keratinocyte basal medium (KBM)-chemically defined (CD) (Lonza, Basel, CH) supplemented with 0.1 mM ethanolamine and 0.1 mM phosphoethanolamine.

Statistical analysis

Statistical analysis was performed using unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test using Prism software (GraphPad, La Jolla, CA). Differences were considered significant when: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr. Annalisa Saltari for her advice and help in the isolation of HPKs. This work was supported by grants from the Wilhelm Sander-Stiftung, the Promedica Stiftung (Chur), the OPO-Stiftung and the Kurt und Senta Herrmann-Stiftung to HDB, the Swiss National Science Foundation (31003A-120400 and 310030-156384) and the Zurich University Research Priority Program "Translational Cancer Research" to LEF, and the Zurich University Forschungskredit Candoc to GF. GF and SG were members of the Life Science Zurich Graduate School.

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FIGURES

Figure 1. Workflow for the generation of CRISPR/Cas9-targeted human primary keratinocytes (HPKs).

(**a**) On day 0, mitotically-inactivated 3T3-J2 feeder cells are seeded at a density of 50%. (**b**) At day 1, freshly isolated HPKs were added (ratio HPKs: J2 5:1) and the medium was changed after 24 hrs (**c**). (**d**) Clones of 5-6 HPKs were transduced with lentiviral vectors encoding sgRNA and Cas9, and the medium was changed after 24 hrs (**e**). (**f**) HPKs were grown until they reached an optimal density of 50-60%; feeder cells were removed by a short incubation with diluted trypsin/EDTA solution (**g**) and keratinocytes were trypsinized and seeded on fresh mitotically-inactivated puromycin-resistant feeder cells. Selection started 24 hrs after splitting. Scale bars, 100 μm; red dashed lines highlight keratinocyte clones.

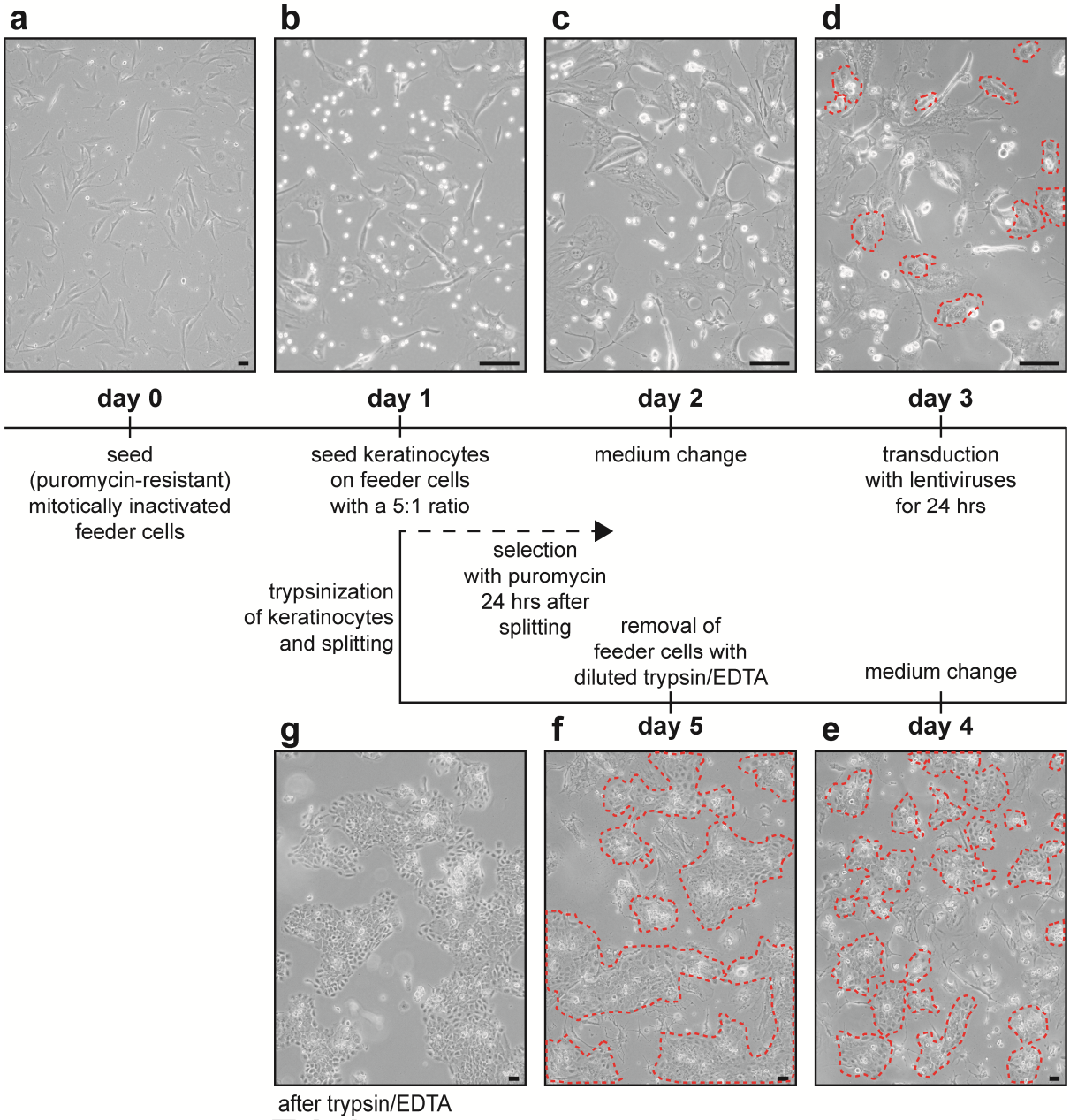
Figure 2. Generation and characterization of CRISPR/Cas9-targeted HPKs.

(**a**) Western blot analysis and (**b**) quantification of ASC bands using lysate of HPKs transduced with a lentiviral vector targeting the ASC gene or a non-targeting sgRNA (control) and cultured under puromycin selection as indicated. (**b**) The intensities of ASC bands on Western blots were normalized to β-actin protein levels and are shown relative to the levels seen in control sgRNA-targeted CRISPR/Cas9 HPKs. sgRNA targeted genes and antibodies used for Western blots are indicated. (**c**) Western blot analysis of cell lysate (CL) and supernatant (SN) of non-targeting control, caspase-1 and ASC sgRNA CRISPR/Cas9-targetd HPKs or wild-type cells maintained on feeder cells. (**d**) ELISA for IL-1β quantification in supernatants of untreated and UVB-irradiated control, caspase-1 and ASC sgRNA CRISPR/Cas9-targetd HPKs and wild-type keratinocytes. (**e**) Western blot analysis using cell lysate of control, caspase-1 and ASC sgRNA CRISPR/Cas9-targetd HPKs and wild-type

HPKs. Cells were harvested at the exponential growth phase (exp), when reaching confluency (conf), both in keratinocyte medium, or after the indicated days in keratinocyte differentiation medium (DM). sgRNA targeted genes and antibodies used for Western blots are indicated. Data are expressed as the mean ± SEM of three independent experiments using two-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test (**d**) or are representative of three independent experiments (**c**).

Figure 3. NLRP1, but not NLRP3, has a key role in sensing UVB radiation and nigericin in HPKs.

Non-targeting control, ASC, NLRP1 (2 sequences) and NLRP3 (2 sequences) CRISPR/Cas9-targeted HPKs were either primed overnight with IFNγ (20 ng/ml) or left untreated. Western blot analysis using cell lysate (CL) of mock-treated cells (**a**). Supernatant (SN) of UVB-irradiated and nigericin-treated cells was analyzed by Western blot for secretion of the indicated proteins (**b**). Quantification of band signal intensity of secreted mature IL-1β and IL-18 normalized to the corresponding untreated samples from three independent experiments (**c** and Supplementary Figure S4). Supernatant (SN) of UVB-irradiated and nigericin-treated cells was analyzed by ELISA for IL-1β secretion levels (**d**) or for LDH release (**e**). sgRNA targeted genes and antibodies used for Western blots are indicated. Data are expressed as the mean ± SEM of four (**d**-**e**) or three (**c**) independent experiments using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test (**d**-**e**) or are representative of three independent experiments (**a**).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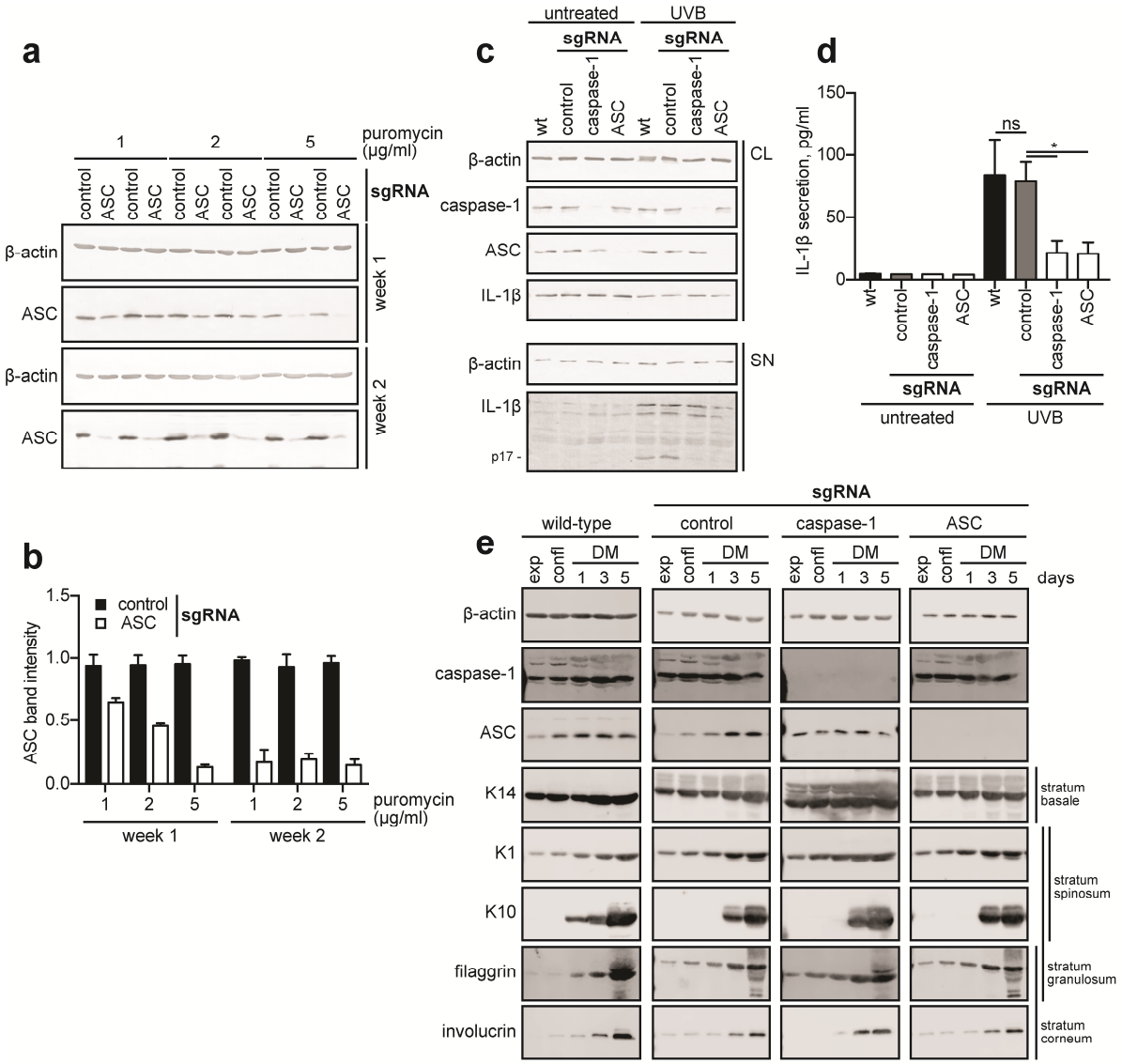
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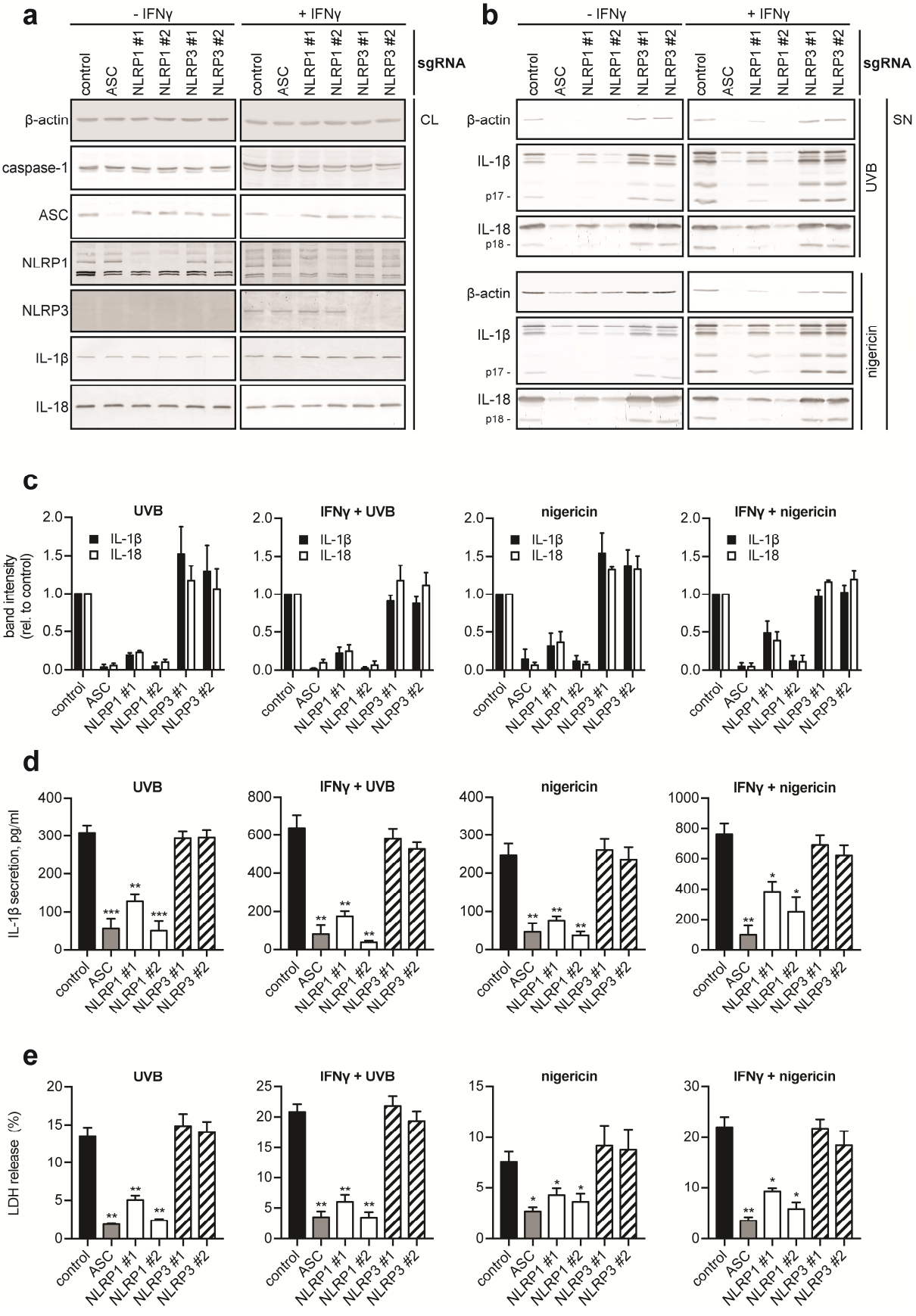
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